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Evaluation of Pretreatment Methods in the Production of Ethanol from Cattail Leaves

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Abstract

Previous research in this lab indicated that cattails are a potential source of biomass for the production of cellulosic ethanol since their carbohydrate composition is comparable to that of other plants being considered for biofuel production. To further test their viability, we tested various pretreatment methods on dried cattail leaves. Before polysaccharides in plants can be enzymatically hydrolyzed to fermentable sugars, the plant material must be pretreated to render the polysaccharides accessible to the enzymes. The purpose of this project has been to compare the efficiency of sulfuric acid and ammonia pretreatment methods in preparing cattail biomass for ethanol production. In this project, dried, powdered cattail leaves were pretreated either by autoclaving them with 2% sulfuric acid for one hour or by incubating them overnight at 40 C° in 15 % aqueous ammonia. Samples of the dried, pretreated solid were treated with cellulase and β -glucosidase for 48 hours. To compare the efficiency of these pretreatment methods, glucose liberated in these samples was measured by a glucose oxidase assay. It was found that more glucose was recovered in the enzymatic hydrolysis (step two) than in the pretreatment step (step one.) In step two, more glucose was liberated from biomass pretreated with ammonia than from biomass pretreated with sulfuric acid. However, more glucose was recovered from in step one by sulfuric acid pretreatment. Overall 27.8 % of the starting biomass was recovered as glucose with ammonia pretreatment compared to 11.7 % for sulfuric acid pretreatment. Interestingly, 22.3% of the starting biomass was recovered as glucose when no pretreatment was used.

Introduction

Volatility in petroleum prices, coupled with the threat of global warming from combustion of fossil fuels, emphasize the necessity of developing alternative energy sources such as renewable biofuels. Currently ethanol is at the center of biofuel research. In the United States, ethanol for fuel is primarily produced by fermentation of corn starch. While this approach is economically beneficial to the agricultural industries, it cannot produce sufficient ethanol to replace our dependence on petroleum and there is debate about whether the use of ethanol derived from corn starch actually reduces carbon emissions. Consequently, considerable research is being conducted to develop procedures for producing ethanol from the abundant cellulose and hemicellulose found in plant biomass. Several plants such as

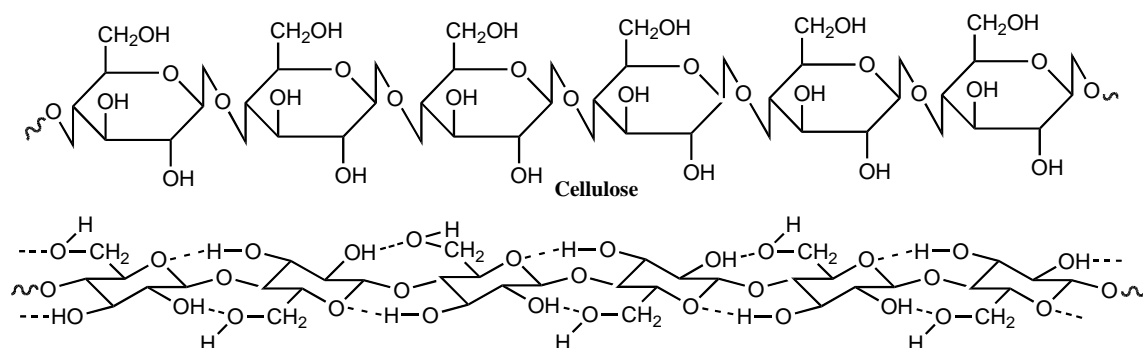
corn stover, switchgrass, aspen and hybrid poplar are under intense investigation as biomass sources.

This report describes an investigation of cattails as a potential source of cellulosic ethanol production. Cattails generate an impressive biomass during a single season; they do not have to be planted annually and do not require cultivation. Development of cattails into a viable source of cellulosic ethanol could provide an incentive for farmers to return cultivated acreage to wetlands.

Petroleum is not an endless resource as is demonstrated by the volatility in gasoline prices. Furthermore, its combustion product, CO₂, contributes to global warming and this has caused great concern in the public. Alternative fuel sources must be found that are cleaner, more renewable and minimize carbon emissions. Ethanol has a great prospective as an alternative fuel. While combustion of ethanol produces CO₂, that CO₂ is in fact recycled when it is taken in by the plants that are used to produce more ethanol. The attractiveness of ethanol comes from the fact that it can be produced here in the United States. This reliance on corn starch as the main source of ethanol has been one factor contributing to increases in food prices. In order to appreciably replace petroleum as fuel, ethanol production must come from sources that are not part of the food supply. In addition, the emission of CO₂ in the production of corn significantly reduces the benefit of recycling the carbon. A different feedstock is needed for ethanol production. A good alternative is plant biomass.

Biomass consists mostly of cellulose, hemicellulose, and lignin. Cellulose composes a major part of plant cell walls. Cellulose is a polymer of glucose linked by β -1,4-glycosidic bonds. These chains are then further linked together by hydrogen bonds between cellulose chains. The following figure shows the structure of cellulose. The top structure is a Haworth representation emphasizing the β -(1-4) glycosidic bonds linking glucose units while the bottom structure reveals the extensive intramolecular hydrogen bonding that contributes to the strength of cellulose fibers.

Figure One



In the plant cell wall, cellulose is typically linked to hemicellulose which is a branched polysaccharide made up of xylose, arabinose, mannose, galactose, and glucose (Hendricks, et al., 2008). Lignin is the third major component of plant cell walls. It is a polymer of phenylpropanes such as p-coumaryl, coniferyl, and sinapyl alcohols. This is the portion of biomass that gives it the structural strength. Cellulose and hemicellulose can be broken down into their respective sugars and then fermented to ethanol. Lignin, however, does not contribute to ethanol production since it contains no sugars. Several plants such as corn stover, switchgrass, aspen and hybrid poplar are under intense investigation as biomass sources (Huang, et al., 2008).

This report describes an investigation of cattails as a potential source of biomass for the production of cellulosic ethanol. Cattails generate an impressive biomass during a single season, they do not have to be planted annually and require no cultivation. Previous research in this lab has shown cattail leaves to be a good source of cellulose and hemicellulose. (Lama, et al., 2007).

Generation of ethanol from any plant biomass requires three steps (Mielenz et al., 2001).

Pretreatment- which helps break up the crystalline structure of biomass so the cellulose and hemicellulose are more accessible.

Enzymatic Hydrolysis- which makes use of enzymes, often from bacteria, to break down the cellulose and hemicellulose into fermentable sugars.

Fermentation- which is usually done by yeast to produce ethanol.

Several different methods of pretreatment have been used to prepare biomass (Hendricks, et al., 2008 and Kim, et al., 2008) The following is a list of some pretreatments:

- Dilute acid- usually sulfuric acid (H_2SO_4)
- Alkaline/solvent- ammonia (NH_4^+), ammonia fiber explosion (AFEX) and other bases
- Steam or liquid hot water- the difference between these is the temperature and pressure used
- Oxidative- uses hydrogen peroxide (H_2O_2) or peracetic acid in an oxidation/reduction reaction with NADH
- Steam explosion- this procedure uses steam for 1-10 minutes until the fibers explode

The goal of this project was to compare the efficiency of dilute acid and aqueous ammonia pretreatments with cattails as the biomass source.

Materials and Methods

I. Pretreatment

A 5.00 g sample of finely ground cattail leaves was placed into each of two 175 ml glass bottles. 75 ml of 2 % H_2SO_4 was added to one sample for dilute acid pretreatment while 75 ml of 15 % NH_4^+ was added to the other for aqueous ammonia pretreatment. The contents were mixed by inversion. The dilute acid sample was autoclaved at 121°C and 15 psi for 60 minutes and the aqueous ammonia sample was placed in a 40°C water bath with shaking for 24 hours.

Following the acid pretreatment, the contents were vacuum-filtered and rinsed with 50 ml of H_2O . The recovered liquids were combined and saved for glucose analysis. Two more washes were done, but discarded. The ammonia pretreatment samples were centrifuged in a Beckman Coulter Centrifuge with a 20.1 rotor at 5000 rpm for 15 minutes. The supernate was saved, the pellet was re-suspended in water and the sample was centrifuged as above. The liquid from both cycles was saved for glucose analysis. Two more water washes were done at the above conditions and wasted. The residual solid was finally washed with water to remove remaining ammonia and recovered by vacuum-filtration. Solids recovered from both pretreatment methods were dried under a vacuum.

The liquids recovered from both pretreatment were neutralized to pH 7. The volumes of both were recorded. The concentration of glucose in these pretreatment liquors was analyzed for glucose as described below.

II. Enzymatic Hydrolysis

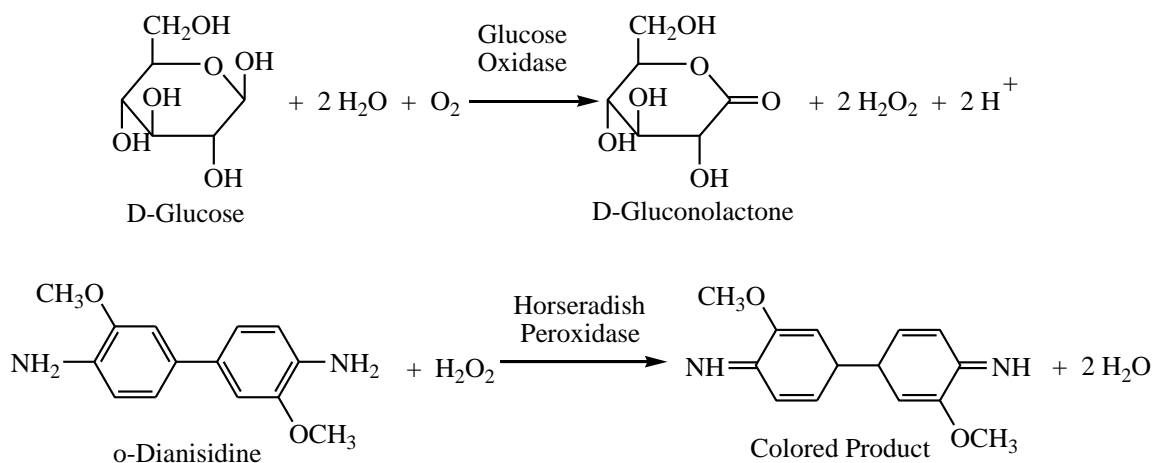
Solids recovered from both pretreatments were ground with a mortar and pestle to a uniform consistency. 2.608 g of ammonia pretreated solid, 1.635 g of dilute acid pretreated solid, and 3.001 g of untreated cattail leaves (as a control) were individually combined with 50 ml of 0.04 M sodium acetate buffer. The pH of the suspension was adjusted to 4.8. Each sample was then brought to a final volume of 75 ml. by the addition of 0.04 M sodium acetate buffer and autoclaved at 121°C and 15 psi for 20 minutes to prevent bacterial contamination. 100 units of cellulase (Sigma) and 15 units of β -glucosidase (Sigma) were added to each suspension. The samples were then placed in a 40°C shaking water bath for 48 hours.

III. Analysis of Glucose

Following enzymatic hydrolysis, glucose concentration was determined in each sample as well as in the liquids recovered from the pretreatment step using a glucose oxidase. This assay is specific for glucose. Standard glucose solutions in the range 0.22 to 1.8 mg.mL were prepared. Samples were filtered prior to analysis. All samples were analyzed in triplicate by combining 100 μ l. sample with 3.0 ml of assay solution containing glucose oxidase, horseradish peroxidase and o-dianisidine (all reagents from Sigma) in 0.1 M sodium phosphate buffer at pH 7.0. After incubating the samples at 37°C for 30 minutes, the each absorbance was measured at 450 nm. The following figure shows the assay reactions.

Figure Two

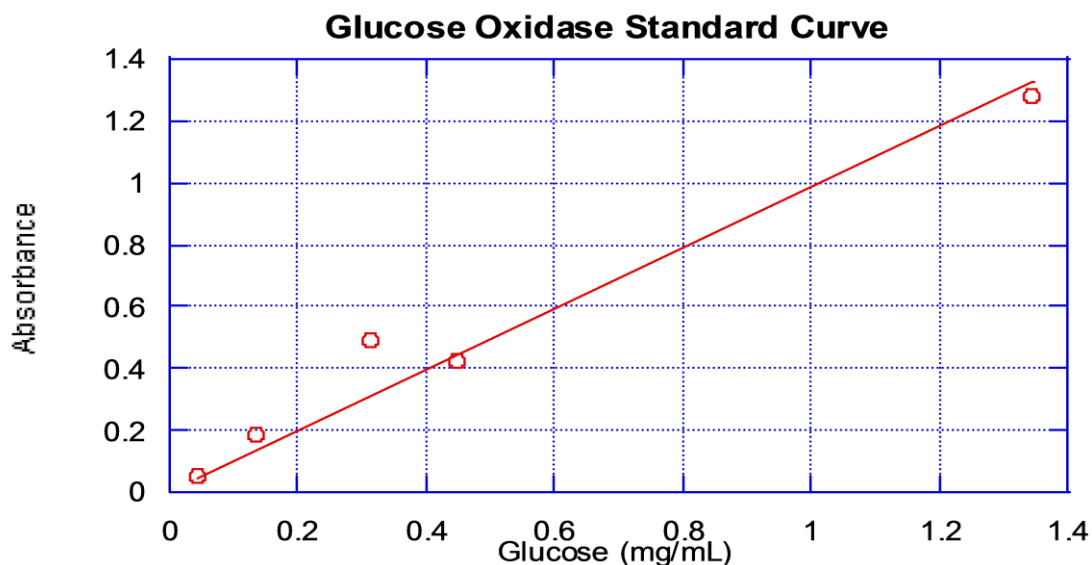
Glucose Oxidase Assay



Results

The glucose analysis yielded the following results based on the standard curves such as that shown in Figure Three. The glucose standard curve used for the liquid recovered during pretreatment was $y = .90888x + .070554$ with an $R^2 = .9864$ where x is the glucose concentration in mg/ml and y is the absorbance at 450 nm. The standard curve for the enzyme hydrolyzed samples was $y = .77656x + .066972$ with $R^2 = .9705$. A typical standard curve obtained by graphing the glucose concentration versus the absorbance at 450 nm is shown below in Figure Three. These standard curve equations can be used to estimate glucose concentrations in the test samples by calculating the glucose concentration that would produce the measured absorbance at 450 nm. The closeness of the variance (R^2) to a perfect value of 1.0000 indicates the standard curves can be used to estimate glucose concentrations in the samples reliably.

Figure Three



The glucose recovered from the enzymatic hydrolysis step is shown in the Figure Four, which compares the amount of glucose recovered as a per cent of the initial dry mass. More glucose is recovered from the ammonia pretreated solid, 25.6 %, than from the sulfuric acid pretreated solid, 7.6 %. A significant amount of glucose, 22.3%, was recovered from the untreated sample. These results are compared in Table One. All measurements were well within the detection limits of the glucose oxidase assay.

These results are consistent with the preliminary characterization of cattail biomass, which indicated that it contained 37.4 % polymeric glucose (Lama, et. al., 2007). Ammonia pretreatment allows recovery of 74 % of the available glucose while sulfuric acid pretreatment allows only 31 % recovery.

Figure Four

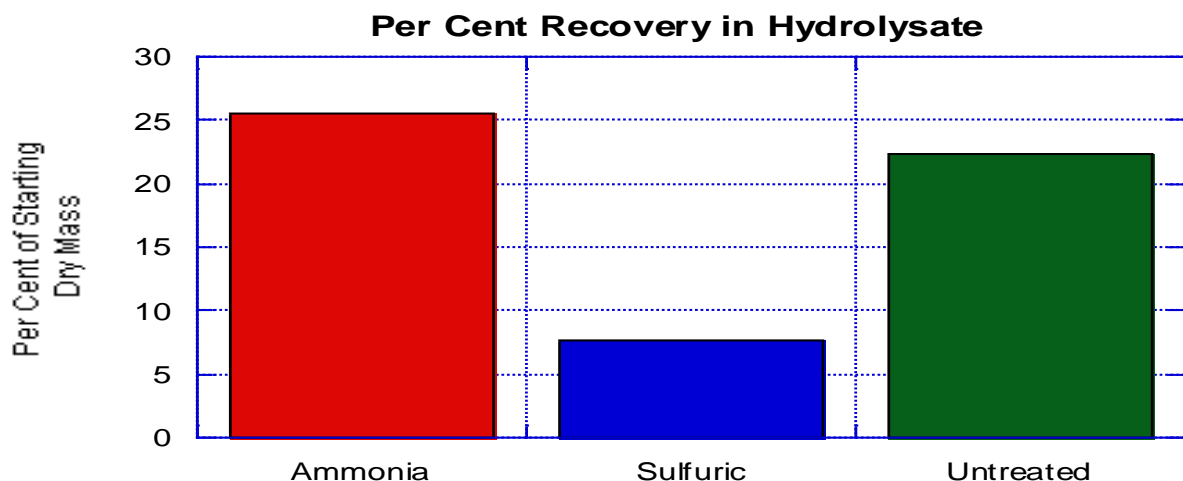


Table One

Comparison of Glucose Recovery

Pretreatment	Per Cent of Starting Mass Recovered as Glucose in Pretreatment Liquor	Per Cent of Pretreated Mass Recovered as Glucose after Enzymatic Hydrolysis	Combined Recovery
Aqueous Ammonia	2.16 +/- 0.09	25.6 +/- 2.2	27.8 +/- 2.3
Dilute Sulfuric Acid	4.11 +/- 0.08	7.6 +/- 0.1	11.7 +/- 0.2
No Pretreatment	Not Applicable	22.3 +/- 2.0	22.3 +/- 2.0

Conclusions

This project revealed the following results.

- More glucose is recovered from the dilute acid pretreatment liquor than from the aqueous ammonia pretreatment liquor.
- Much more glucose is recovered by enzymatic hydrolysis from the aqueous ammonia pretreated solid than from the dilute acid pretreated solid.
- Overall, more glucose is recovered from the aqueous ammonia pretreatment than from the dilute acid pretreatment
- Unexpectedly, the untreated solid produced more glucose than did the solid pretreated with dilute H₂SO₄.

The final observation raises the possibility that a simple pretreatment with acetate buffer avoiding the use of either sulfuric acid or ammonia may be effective. This would reduce expense and environmental impact. Degradation of glucose in acidic conditions as well generation of inhibitors of the enzymatic hydrolysis are likely explanations for the low recovery of glucose observed in the sulfuric acid pretreatment.

These results indicate that investigation of cattail biomass as an alternative feedstock to corn starch for ethanol production should be continued.

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